Histone deacetylase 1 regulates tissue destruction in rheumatoid arthritis

Sarah Hawtree1, Munitta Muthana1, J. Mark Wilkinson2, Mohammed Akil1 and Anthony G. Wilson1,3,*

1Department of Infection and Immunity and, 2Academic Unit of Bone Metabolism, University of Sheffield, Sheffield S10 2RX, UK and 3Conway Institute, Belfield Campus, University College Dublin, Dublin 4, Ireland

*To whom correspondence should be addressed at: Conway Institute, Belfield Campus, University College Dublin, Dublin 4, Ireland. Tel: +353 17166801; Email: gerry.wilson@ucd.ie

Abstract
Emerging evidence implicates epigenetic mechanisms in the pathogenesis of rheumatoid arthritis (RA). In this study, we have investigated the role of histone deacetylase (HDAC) enzymes in RA synovial fibroblasts (RASFs), a key cellular mediator of cartilage and bone destruction and determined effects of HDAC1 inhibition on both RASF phenotype in vitro and joint inflammation and damage in the collagen-induced arthritis (CIA) model. Expression of HDACs 1–11 messenger ribonucleic acid (mRNA) was compared between RASFs and osteoarthritic synovial fibroblast (OASFs) using quantitative polymerase chain reaction. HDAC1 expression in RASFs was inhibited using small interfering RNA (siRNA) technology to assess effects on invasiveness, migration, proliferation and apoptosis. Effects of HDAC1 knockdown (KD) on the transcriptome were assessed using gene microarrays. The effects of siRNA-mediated HDAC1KD on clinical scores, tissue inflammation and damage were assessed on CIA up to 47 days following immunization. Expression of HDAC1 was significantly higher in RASFs than OASFs. HDAC1KD resulted in reduced proliferation, invasion and migration in vitro and transcriptome profiling revealed effects on expression of genes regulating proliferation migration and inflammation. Furthermore, inhibition of HDAC1 in CIA resulted in reduced joint swelling, cartilage and bone damage and lower tumor necrosis factor in joint tissue. These results implicate HDAC1 as an important mediator of tissue damage in RA and support the potential therapeutic utility of inhibitors of this enzyme.

Introduction
Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation in synovial joints frequently resulting in cartilage and bone damage. The etiology is multifactorial with >100 susceptibility loci identified in recent genome wide studies (1). Smoking is the major environmental risk factor (2), whilst alcohol consumption reduces both RA risk and severity (3). The characteristic pathology is increased thickness of the synovial lining layer consisting of macrophages and RASFs, whilst the sub-lining infiltration is composed of inflammatory cells including T and B cells, macrophages, blood vessels and fibroblasts (4).

Macrophages are believed to be key mediators of tissue damage principally by the production of pro-inflammatory cytokines including tumor necrosis factor (TNF), IL-1, IL-6 and IL-8. The RASF is also a central mediator of tissue destruction producing many disease-relevant mediators including cytokines, chemokines, adhesion molecules and proteases (5). It has a semi-transformed phenotype in vitro with loss of contact inhibition, high proliferative activity and resistance to apoptosis (6). Engraftment of normal human cartilage along with RASFs into severe combined immunodeficiency (SCID) mice reveals this aggressive phenotype to be maintained for up to 60 days and to be independent of T cells and other adaptive immune cells (7).
Furthermore, purified RASFs can transfer arthritis if injected into the knee joint of SCID mice (8).

Epigenetics mechanisms have been implicated in the pathogenesis of RA. Levels of DNA methylation are lower in RASFs than synovial fibroblasts from OA (OASFs) and treatment of the latter with a DNA demethylating agent results in a switch to a RASF-like phenotype with upregulation of growth factors and receptors, extracellular matrix proteins, adhesion molecules and matrix-degrading enzymes (9). A DNA methylene array study of 485k CpG motifs reported differential methylation of 1879 loci, with hypomethylation in pathways associated with cell migration including cell adhesion, transendothelial migration and extracellular matrix interactions (10). The expression of the retrotransposon, long interspersed element 1 (LINE1), is 30–300 times higher in RASFs and increases upon treatment with a DNA demethylating agent (11), and has been shown to induce p38 mitogen-activated protein kinase, an important regulator RASF activation characterized by the induction of IL-6 and IL-8 expression (12). The acetylation of histone proteins is regulated by the relative activities of two enzyme families; histone acetyltransferases (HATs) and histone deacetylases (HDACs). Total HAT activity is similar in RA and OA synovial tissue, however the data for overall HDAC activity and histone acetylation status in synovial tissue are conflicting (13–15). Similarly discrepancies in expression of individual HDAC proteins have been reported with lower HDAC1 and 2 levels described in nuclear extracts of synovial cells (13); however opposite findings have been reported in synovial fibroblasts (16) and synovial cell nuclear extracts (14). Similar discrepant results have also been reported for overall HDAC activity in blood mononuclear cells from RA cases compared with healthy controls (17,18).

Here we identify HDAC1 as a key mediator of the autoaggressive RASF phenotype. It is highly expressed in RASFs compared with OASFs in vitro and in vivo, and targeting expression using siRNA technology resulted in attenuated invasiveness, proliferation and migration of RASFs in vitro as well as modulated expression of genes in RA-relevant pathways. Furthermore targeting HDAC1 in the CIA model of RA resulted in attenuated joint inflammation and cartilage and bone damage, and reduced TNF but higher IL-10 expression.

**Results**

**High HDAC1 expression in RASFs**

Levels of HDAC1–11 mRNA were compared between RASFs (n = 10) and OASFs (n = 7) using quantitative PCR. All HDACs, except HDAC4, were higher in the former, with HDAC1 being greatest and statistically significant (6-fold, P = 0.03) (Fig. 1A), this was confirmed at the protein level using western blot and densitometry (Fig. 1B). Immunohistochemistry confirmed HDAC1 was present throughout RA synovial tissue, whereas an IgG isotype showed no non-specific staining (Fig. 1C). Quantification analysis of HDAC1-positive cells confirmed expression to be significantly higher in RA compared with OA synovium (P = 0.005) (Fig. 1D). Immunofluorescence confocal microscopy revealed much higher expression of HDAC1 (red) in RASFs (green) from RA compared with OA, and was both cytoplasmic and nuclear (blue) (Fig. 1E).

**Inhibition of HDAC1 attenuates RASF phenotype in vitro**

We used siRNA to target HDAC1 expression in RASFs, this resulted >60% reduction in mRNA and protein expression assayed by both western blot and confocal microscopy (Figure S1A–C), levels of HDAC2, which is highly homologous to HDAC1, were not reduced. There were modest changes in HDAC9 (30%) and HDAC10 (20%) mRNA expression (Supplementary Material, Figure S2). Cells from 6 RA patients were transfected with non-targeting control (NTC)- or HDAC1-targeting siRNA. Proliferation was assessed by thymidine incorporation and was significantly reduced by HDAC1KD (P < 0.05) (Fig. 2A), there was however no effect on apoptosis (P = 0.96) (Fig. 2B and Supplementary Material, Figure S3A). The scratch assay was used as a measure of cell migration and was significantly attenuated by HDAC1KD; after 24 h a slower rate of closure is observed in the HDAC1KD cells compared with NTC (35 ± 5.8%; 46 ± 5.7% respectively, P = 0.01) (Fig. 2C and Supplementary Material, Figure S3B), suggesting that HDAC1 contributes to the migration of RASFs. To ascertain that this was not the result of altered proliferation, the assay was repeated in the presence mitomycin C; this confirmed that the difference in migration was independent of proliferative rate (data not shown). We next determined effects on RASF invasiveness using the Matrigel assay (Supplementary Material, Figure S3C). After 48 h the number of cells within the gel were counted in 10 fields of view; HDAC1KD significantly reduced the invasiveness of RASFs (average number of cells = 10.9 ± 6.4) compared with NTC (average number of cells = 18.5 ± 7.7) (Fig. 2D, P = 0.02). These data indicate that HDAC1 plays a significant role in regulating the major features of the autoaggressive phenotype of RASFs.

**HDAC1 regulates pathways involved in RASF-related pathology**

As HDAC1 is known to be an important master regulator of gene expression we next determined the effects of its inhibition on the transcriptome. Paired RASFs samples (n = 3) were transfected with NTC- or HDAC1-targeting siRNAs and RNA was harvested 24 h later. Prior to performing the assays, the levels of HDAC1 mRNA reduction were determined relative to NTC in each paired sample and were 37, 47 and 51%. In the analysis the effects of HDAC1KD on each transcript were averaged across the three samples and the data displayed in a volcano plot (Fig. 2E); 174 genes were significantly up-regulated and 76 genes down-regulated (P ≤ 0.01). Hierarchical clustering, ranked on enrichment scores, revealed eight functional categories (Fig. 1F and Supplementary Material, Table S1) and included transcripts associated with proliferation (e.g. SOS1 and NAMPT) and migration (e.g. VEGFA and FGF2) and inflammation (e.g. IL-1R2 and CD59) (Table 1). To validate the microarray results, a selection of these genes were analyzed using RT-qPCR (Supplementary Material, Figure S3D).

**Regulation of HDAC1 in RASF**

We determined how TNF and other inflammatory modulators might influence HDAC1 expression in RASF. Cells were incubated with TNF (50 ng/ml), hypoxia (0.1% O2), lipopolysaccharide (100 µg/ml), dexamethasone (1 x 10–6 M) or hypoxia and TNF for 4 or 24 h and HDAC1 mRNA analysis by RT-qPCR. At both timepoints combined exposure to hypoxia and TNF caused a significant reduction in HDAC1 expression compared with an untreated control (Supplementary Material, Figure S4A and B). After 24 h culture in hypoxia alone also resulted in significantly reduced HDAC1 expression.

**Inhibition of HDAC1 reduces inflammation and tissue damage in CIA**

Having established HDAC1 as a regulator of RASF invasion and migration, we hypothesized that HDAC1KD would attenuate the characteristic pathology seen in the CIA model of inflammatory
Injection of HDAC1-targeting siRNA resulted in lower expression of HDAC1 in all tissues of DBA/1 mice with significant suppression in paws, liver and lungs (Supplementary Material, Figure S5). To test our hypothesis, mice were administered with three doses of 5 mg/kg NTC- or HDAC1-targeting siRNA by tail vein injection. Mice were monitored daily; paw inflammation...
developed in all NTC-treated animals after 24 days and became increasingly pronounced by week 7, HDAC1KD mice had markedly attenuated paw inflammation that was statistically significant different from NTC-treated mice by 28 days (Fig.3A). Histological scores of inflammation were significantly reduced in the HDAC1KD group (P = 0.01) (Fig. 3B), with reduced cartilage loss (P = 0.01) (Fig. 3C) and bone damage (P = 0.001) (Fig. 3D) in hind paws. A particularly striking finding was reduced expression of TNF in the joints of HDAC1KD animals, conversely levels of the immunomodulator cytokine, IL-10, were higher (Fig. 3E).

Discussion

Although there is substantial evidence showing benefits of HDACi on the RASF phenotype in vitro and in animal models of RA the relative contribution of individual HDACs is poorly understood. The use of a specific HDAC1 KD model allowed us to investigate the effects on paw inflammation and joint tissue damage. The results support the notion that HDAC1 plays a critical role in the pathogenesis of RA, and suggest that targeting HDAC1 may be a promising therapeutic strategy for this disease.
understood. In this study, we report evidence that implicates HDAC1 as an important regulator of tissue damage in RA. We show that HDAC1 inhibition in RASFs results in attenuation of the autoaggressive phenotype that is characterized by reduced metalloproteinase production and lower proliferative rate (19–21), while the predominant effect on synovial macrophages is the induction of apoptosis (22). Their use in murine models of inflammation and tissue damage (19,21) has established the importance of HDACs in RA. The importance of HDACs in RA has been established in studies using inhibitors of Class I HDAC (HDAC1, 2, and 8); for example, incubation of RASFs with these agents results in increased apoptosis, reduced production of IL-6 and matrix metalloproteinase production and lower proliferative rate (19–21), while the predominant effect on synovial macrophages is the induction of apoptosis (22). Their use in murine models of RA has also proven effective at reducing joint inflammation and damage, and lowering production of pro-inflammatory cytokines (23–25). The superior efficacy of MS-275 in CIA has been proposed to be due to its greater specificity for Class I HDACs.

The HDAC1KD samples were compared with NTC controls using the Lumi software in the statistical package. All the significant genes (P < 0.01) were analyzed using DAVID software and categorized into clusters of biological functions. Those clusters with the highest enrichment scores are shown here along with their fold change (FC) and P-value.

### Table 1. Hierarchical clustering of differentially expressed genes following HDAC1KD in RASFs

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Gene</th>
<th>Gene ID</th>
<th>Title</th>
<th>Fold change</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Apoptosis</td>
<td>IFI6</td>
<td>NM_022873.2</td>
<td>Interferon, α-inducible protein 6</td>
<td>1.36</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>B2M</td>
<td>NM_004048.2</td>
<td>β-2-microglobulin</td>
<td>1.22</td>
<td>0.0093</td>
</tr>
<tr>
<td></td>
<td>DYRK2</td>
<td>NM_003583.2</td>
<td>Dual-specificity tyrosine-(Y)-phosphorylated kinase 2</td>
<td>−1.39</td>
<td>0.0003</td>
</tr>
<tr>
<td>2. Regulation of Glucose Metabolism</td>
<td>GS3K</td>
<td>NM_019884.2</td>
<td>Glycogen synthase kinase 3α</td>
<td>1.23</td>
<td>0.0057</td>
</tr>
<tr>
<td></td>
<td>GAPDH</td>
<td>NM_014364.3</td>
<td>Glycerophosphate dehydrogenase, spermatic activity</td>
<td>1.21</td>
<td>0.0093</td>
</tr>
<tr>
<td></td>
<td>DYRK2</td>
<td>NM_003583.2</td>
<td>Dual-specificity tyrosine-(Y)-phosphorylated kinase 2</td>
<td>−1.39</td>
<td>0.0003</td>
</tr>
<tr>
<td>3. Circulatory System Processes</td>
<td>TPM1</td>
<td>NM_001018008.1</td>
<td>Tropomyosin 1 (α)</td>
<td>1.21</td>
<td>0.0081</td>
</tr>
<tr>
<td></td>
<td>GUCA2B</td>
<td>NM_007102.1</td>
<td>Guanylate cyclase activator 2B (uroguanylin)</td>
<td>1.22</td>
<td>0.0087</td>
</tr>
<tr>
<td></td>
<td>RGCN1</td>
<td>NM_203417.1</td>
<td>UV radiation resistance associated gene</td>
<td>1.30</td>
<td>0.0014</td>
</tr>
<tr>
<td></td>
<td>MAPK14</td>
<td>NM_139013.1</td>
<td>Mitogen-activated protein kinase 14</td>
<td>1.26</td>
<td>0.0043</td>
</tr>
<tr>
<td>4. DNA Metabolism and Repair</td>
<td>MBD4</td>
<td>NM_003925.1</td>
<td>Methyl-CpG binding domain protein 4</td>
<td>1.26</td>
<td>0.0073</td>
</tr>
<tr>
<td></td>
<td>HMGBl</td>
<td>NM_002128.4</td>
<td>High-mobility group box 1</td>
<td>−1.23</td>
<td>0.0098</td>
</tr>
<tr>
<td></td>
<td>DNM1T1</td>
<td>NM_001379.1</td>
<td>DNA (cytosine-5′-)methyltransferase 1</td>
<td>−1.26</td>
<td>0.0027</td>
</tr>
<tr>
<td>5. Regulation of transcription factor activity</td>
<td>EDA2R</td>
<td>NM_021783.2</td>
<td>Ectodysplasin A2 receptor</td>
<td>1.40</td>
<td>0.0013</td>
</tr>
<tr>
<td></td>
<td>PRDX2</td>
<td>NM_181738.1</td>
<td>Peroxiredoxin 2, nuclear gene encoding mitochondrial protein</td>
<td>−1.23</td>
<td>0.0083</td>
</tr>
<tr>
<td>6. Cell proliferation</td>
<td>CLU</td>
<td>NM_203339.1</td>
<td>Clusterin</td>
<td>1.25</td>
<td>0.0078</td>
</tr>
<tr>
<td></td>
<td>LAMC1</td>
<td>NM_002293.2</td>
<td>Laminin, gamma 1 (formerly LAMB2)</td>
<td>1.24</td>
<td>0.0039</td>
</tr>
<tr>
<td></td>
<td>SOS1</td>
<td>NM_005633.2</td>
<td>Son of sevenless homolog 1 (Drosophila)</td>
<td>−1.23</td>
<td>0.0058</td>
</tr>
<tr>
<td></td>
<td>NAMPT</td>
<td>NM_005746.2</td>
<td>Nicotinamide phosphoribosyltransferase</td>
<td>−1.28</td>
<td>0.0019</td>
</tr>
<tr>
<td>7. Extracellular matrix</td>
<td>COL1a2</td>
<td>NM_00089.3</td>
<td>Collagen, type I, α2</td>
<td>1.35</td>
<td>0.0022</td>
</tr>
<tr>
<td></td>
<td>FBLN2</td>
<td>NM_001998.2</td>
<td>Fibulin 2</td>
<td>1.31</td>
<td>0.0012</td>
</tr>
<tr>
<td></td>
<td>BMP8B</td>
<td>NM_001720.3</td>
<td>Bone morphogenetic protein 8b</td>
<td>1.28</td>
<td>0.0088</td>
</tr>
<tr>
<td></td>
<td>COMP</td>
<td>NM_00095.2</td>
<td>Cartilage oligomeric matrix protein</td>
<td>1.27</td>
<td>0.0032</td>
</tr>
<tr>
<td></td>
<td>CLU</td>
<td>NM_203339.1</td>
<td>Clusterin</td>
<td>1.25</td>
<td>0.0078</td>
</tr>
<tr>
<td>8. Migration</td>
<td>VEGFA</td>
<td>NM_00025366.1</td>
<td>Vascular endothelial growth factor A, transcript variant 1</td>
<td>−1.22</td>
<td>0.0065</td>
</tr>
<tr>
<td></td>
<td>FGF2</td>
<td>NM_002006.3</td>
<td>Fibroblast growth factor 2 (basic)</td>
<td>−1.24</td>
<td>0.0062</td>
</tr>
<tr>
<td></td>
<td>VEGFA</td>
<td>NM_00025367.1</td>
<td>Vascular endothelial growth factor A, transcript variant 3</td>
<td>−1.26</td>
<td>0.0085</td>
</tr>
<tr>
<td>9. Other</td>
<td>FOSB</td>
<td>NM_006732.1</td>
<td>FBJ murine osteosarcoma viral oncogene homolog B</td>
<td>1.90</td>
<td>8.67E−08</td>
</tr>
<tr>
<td></td>
<td>KIA1199</td>
<td>NM_018689.1</td>
<td>Hyaluronan binding protein</td>
<td>1.34</td>
<td>0.0047</td>
</tr>
<tr>
<td></td>
<td>FAM19A3</td>
<td>NM_00004440.1</td>
<td>Family with sequence similarity 19 (chemokine (C-C motif)-like)</td>
<td>1.32</td>
<td>0.0066</td>
</tr>
<tr>
<td></td>
<td>IIL2</td>
<td>NM_004633.3</td>
<td>Interleukin 1 receptor, type II</td>
<td>1.22</td>
<td>0.0090</td>
</tr>
<tr>
<td></td>
<td>CD59</td>
<td>NM_203331.1</td>
<td>CD59 molecule, complement regulatory protein</td>
<td>1.22</td>
<td>0.0093</td>
</tr>
<tr>
<td></td>
<td>HAS2</td>
<td>NM_00528.1</td>
<td>Hyaluronan synthase 2</td>
<td>−1.32</td>
<td>0.0015</td>
</tr>
<tr>
<td></td>
<td>MED28</td>
<td>NM_025205.3</td>
<td>Mediator complex subunit 28</td>
<td>−1.60</td>
<td>3.24E−06</td>
</tr>
</tbody>
</table>

The HDAC1KD samples were compared with NTC controls using the Lumi software in the statistical package. All the significant genes (P < 0.01) were analyzed using DAVID software and categorized into clusters of biological functions. Those clusters with the highest enrichment scores are shown here along with their fold change (FC) and P-value.
Figure 3. HDAC1KD attenuates joint inflammation and damage in CIA. DBA/1 mice induced with CIA were treated with either HDAC1-targeting or NTC siRNA (5 mg/kg) with n = 10 mice/group. (A) HDAC1KD significantly attenuated the clinical phenotype of CIA in DBA/1 mice. Representative front and hind paw images from each group are shown. At Day 49 hind paws were removed for histological and imaging studies. (B) Representative histological H&E stained sections are shown and were quantified by individually scoring synovial hyperplasia, inflammation, pannus formation and bone loss. (C) Sections were stained with safranin O-fast green to determine the loss of proteoglycans. Safranin O-positive joints were counted out of total cartilaginous joints. (D) Bones were scanned on a MicroCT scanner at 4.3 μm resolution and bone volume was analyzed over a 2 mm section of the third metatarsophalangeal articulation using the CTAn software. (E) RT-PCR of hind paws shows the relative mRNA expression of cytokine genes in paws of HDAC1KD compared with NTC-treated mice. Data are the mean plus SD, with the dotted line representing no change from NTC control. Analysis was carried out using a one-sample t-test against a theoretical mean of 1 (*P < 0.05).
particularly HDAC1 (23). A preliminary study has reported that Givinostat, a Class I and II HDACi, is both efficacious and safe in a 12 week trial involving 17 patients with systemic-onset juvenile idiopathic arthritis (26).

Our gene expression data confirm the role of HDAC1 in regulating expression of genes that have been implicated in the pathogenesis of RA. Both vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2) have been implicated in RA, in addition, FGF2 stimulates both RASF migration and induces expression of RANKL with consequent osteoclastogenesis and bone damage (27). Both IL-1RII and CD59 have anti-inflammatory properties with deletion of CD59 resulting in increased tissue damage in a murine RA model (28). Son of sevenless (SOS1) has not previously been implicated in RASF biology, although it is important in immune cell signaling and is upregulated in peripheral blood T cells of RA cases compared with those of controls (29), although it is potential interesting that hereditary gingival fibromatosis has been linked with mutations in SOS1 (30).

The interplay between inflammation and HDACs in the RA joint is poorly understood. Our data show that HDAC1 inhibition reduces TNF production in the inflamed joint of the CIA model. Levels of HDAC1 have been positively correlated with TNF expression in RA synovium (15), and TNF treatment of RASFs increases total HDAC activity particularly HDAC1 (14), furthermore the Class 1 HDAC inhibitor, Trichostatin A, has been shown to inhibit HDAC1 binding to the TNF promoter resulting in increased expression of this pivotal cytokine in RA (31). This suggests an important positive feedback loop in RA involving HDAC1 and TNF, which may propagate chronic inflammation and joint damage and could be an important therapeutic target.

Although the main mechanism by which HDACs regulate gene expression is by the alteration of the histone signature, the activities of many non-histone proteins, including nuclear factor-κB and the glucocorticoid receptor, are influenced by acetylation by individual HDACs (32). Thus to fully understand how HDAC1 modulates the behavior of RASFs it will be necessary to understand its activity on the cellular acetylome including non-histone proteins. Intuitively, inhibition of HDAC1 should result in higher gene expression as acetylation is a permissive epigenetic mark, however it is likely that effects on regulatory networks is the mechanism of reduced mRNA expression of a significant number of genes, a time-course of HDAC1 KD effects on the transcriptome would be informative.

The severity of joint destruction in RA is highly variable between patients and is known to have a genetic component (33), but is also associated with environmental exposures such as smoking (34) and alcohol consumption (3). Smoking is the major environmental risk factor for RA and has been associated with specific epigenetic changes in peripheral blood leukocytes (35) and with more severe radiological damage (35). For these reasons it will for these reasons be important to compare the epigenetic signature in RASFs from smokers and non-smokers. The invasiveness of RASFs in vitro has been correlated with the rate of radiological progression of joint damage (36), and studies to characterize the genetic and epigenetic variants that underpin the variability in this cellular phenotype should give useful prognostic information. This should facilitate patient stratification, and also potentially identify epigenetic pathways that could be therapeutically targeted to improve the outcome of RA patients. Our data suggest that specific inhibition of HDAC1 is one such target.

**Materials and Methods**

**Synovial samples**

Synovial samples were obtained at arthroscopy or large joint arthroplasty and were approved by the South Yorkshire Research Ethics Committee (SSREC/03/106) and the Oxford Research Ethics Committee C (Reference 10/H0606/20). Written informed consent was obtained from all participants according to the approved protocol. The diagnosis of RA was based on the 1987 American College of Rheumatology criteria (37). Briefly, arthroscopy and synovial biopsy was performed, under anesthesia and sterile conditions, using a Storz 2.7 mm needle arthroscope (Storz, Tuttlingen, Germany) and a 2 mm grasping forceps as previously described by us (38). Biopsy samples were obtained from all compartments of the knee joint, embedded in Tissue-Tek OCT compound (Sakura Finetek, Zoeterwoude, The Netherlands), snap frozen and stored in liquid nitrogen for future immunofluorescence staining.

**Isolation and culture of RASF**

Fresh synovial biopsies were digested using 1 mg/ml collagenase from Clostridium histolyticum (Sigma Aldrich, MO, USA). Dissociated cells were grown to confluence (10 days) in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, MA, USA), 10% fetal bovine serum (Biosera, East Sussex, UK), 1% penicillin-streptomycin (Lonza-Biowhittaker, Cologne, Germany) before trypsinization and passage. Passages 3–8 of the RASF were used in subsequent experiments, at which time they were a homogeneous population of fibroblasts (38).

**Real-time PCR**

Total mRNA was extracted from cultured RASF (1 × 10⁶ cells) or mouse tissues using the RNasy Mini Kit (Qiagen, Duesseldorf, Germany). Reverse transcription of 1 μg total mRNA was carried out using the Nanoscript Reverse Transcription Kit (Primer Design, Southampton, UK). For murine bones RNA extraction was performed using Trizol (Life Technologies). PCR amplification of complementary DNA was performed using SYBR green mastermix (Primers design) and 10 μm sense and antisense primers (Supplementary Material, Table S1). Reactions were processed in a DNA thermal cycler (ABI Prism 7900HT Sequence Detection System, Life Technologies) with 50 cycles of 15 s of denaturation at 95°C, and 60s of annealing at 60°C, followed by 50s of elongation at 60°C. The Ct values generated from these samples were normalized to β-actin. In order to calculate the absolute concentration of HDAC1 in the sample, a standard curve was created using a serial dilution of a HDAC1 plasmid clone (Source BioScience, Nottingham, UK) at a known concentration of 26.8 ng/μl. A standard curve was created using Microsoft Excel and a line equation for the HDAC1 standard curve was \( y = -1.706\ln(x) + 9.886 \). This equation was used to convert all the Ct values in the study to absolute concentrations.

**Immunohistology**

Tissue obtained at arthroscopy from the knees of individuals with RA was fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (5 μm) were mounted onto glass slides, dewaxed and rehydrated through an alcohol series before antigen retrieval was achieved by heating in trisodium citrate (pH 6.0) for 10 min in a high-powered microwave. Blocking was carried out using horse serum (Vector Laboratories, CA, USA), and this was followed by incubation with a 1 in 200 dilution of anti-
HDAC1 antibody (Millipore) for 1 h. Controls were incubated in the same dilution of matched IgG (Vector Laboratories). Bound antibody was detected using an avidin–biotin complex (ABC) peroxidase kit (Vector Laboratories). Briefly, 50 µl of biotinylated secondary antibody was added for 30 min, then ABC peroxidase was added for a further 30 min. Peroxidase activity was then measured using a 3,3-diaminobenzidine substrate. All slides were counterstained with Gills hematoxylin (Sigma Aldrich).

**In vitro HDAC1 siRNA-mediated knockdown**

In order to compare the biological effects of HDAC1 on the synovial fibroblasts we used small interfering RNA technology (siRNA) to knockdown gene expression. RASF seeded at 2–5 × 10^4 cells per well were transfected with 25 nM HDAC1 or NTC smartpool siRNA using Dharmfect 4 (ThermoScientific, Loughborough, UK). Sequences of siRNA molecules are listed in Supplementary Material, Table S2. The cells were incubated at 37°C and 5% CO₂ for 24–72 h. Immunofluorescence microscopy, western blotting and real-time PCR were used to assess HDAC1 mRNA and protein expression.

**Immunofluorescence staining**

Frozen synovial tissue sections (5 µm) were fixed in 4% paraformaldehyde and then co-incubated with 1 in 25 diluted antibodies against mouse anti-fibroblast surface protein (Abcam, Cambridge, UK) and with rabbit polyclonal anti-HDAC1 for 1 h followed by addition of Alexa Fluor 488-conjugated anti-mouse and Alexa Fluor 568-conjugated anti-rabbit (Life Technologies). Slides were placed in Vectorshield mounting medium containing DAPI (Vector Laboratories). Sections incubated for 1 h with an isotype-matched control were used as negative controls. Images were collected using a confocal microscope (LSM510; Zeiss, Oberkochen, Germany). The background fluorescence level was set with the negative controls, and images were analyzed using Zen image analysis software 2009 (Zeiss).

**Western blotting**

Whole-cell lysates from cultured RASF were prepared from 2 × 10⁶ cells by homogenization in MPer Mammalian protein extraction buffer with a protease inhibitor cocktail (ThermoScientific). A murine embryonic fibroblast lysate was used as a positive control (Abcam). Protein concentrations were determined by NanoDrop (ThermoScientific), and normalized to 1 mg/ml. Protein samples were separated on 12% pre-cast sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels (BioRad, Hertfordshire, UK) and transferred onto a nitrocellulose membrane using the iBlot machine with western blot transfer stacks (Life Technologies). The membrane was viewed at 37°C and 5% CO₂ for 40 min. After further washes, the hybridized bands were detected using an ECL plus detection kit (GE Healthcare, Chalfont St. Giles, UK). The membrane was viewed against negative controls was <0.05 for all hybridizations. The expression values were then adjusted with the variance stabilizing transformation method and then normalized with a quantile

**Thymidine incorporation assay**

RASF proliferation was assessed by [3H]thymidine incorporation; following siRNA treatment plates were pulsed with 1 µCi/well [3H]thymidine (Perkin Elmer, MA, USA) for 24 h. Cells were washed with PBS, incubated with 10% w/v TCA at 4°C for 10 min, washed and incubated with sterile 1 M NaOH overnight at 37°C. The cells were transferred to scintillation fluid (Perkin Elmer) and [3H]thymidine uptake was measured in an LS 6500 scintillation counter (Beckman Coulter, CA, USA). Statistical analysis was carried out using a paired t-test (n = 6).

**Scratch wound assay**

The migration of cells was measured in a scratch-wound assay in which the RASF migrate into an area that has been mechanically denuded of cells. Following siRNA treatment, RASF were plated at a concentration of 2 × 10⁴ cells/well and a wound was introduced in a straight line across the cells with a p200 pipette tip in each well of a 96-well plate. The width of the streak was measured manually using Image-J software and an inverted microscope (Leica, Wetzlar, Germany) after 0, 24 and 48 h. RASF migration was calculated using the formula for gap closure, [(width at 0 h) – (width at 24 h)]/(width at 0 h) × 100%. Statistical analysis was carried out using a paired t-test (n = 6).

**Invasion assay**

In vitro invasion of RASF was assayed in a transwell system using the BD BioCoat™ BD Matrigel™ Invasion Chamber (BD Biosciences, NJ, USA). Briefly, following siRNA treatment cells were harvested by trypsin–ethylenediaminetetraacetic acid digestion. Subsequently 1.0 × 10⁴ cells were resuspended in 500 µl of serum-free DMEM and plated in the upper compartment of the rehydrated Matrigel-coated inserts. The lower compartment was filled with complete media and the plates were incubated at 37°C for 48 h. Cells were removed from the upper well and the surface of the insert was then wiped with a cotton swab to remove non-invading cells in to the Matrigel layer. The insert was fixed in 100% methanol and was stained with Hematoxylin & Eosin (Sigma Aldrich) and the total number of cells that had invaded through Matrigel counted at ×40 magnification. To assess the average number of migrating cells, cells were counted in 10 random high-power fields (five centrally, and five peripherally). Statistical analysis was carried out using a paired t-test (n = 6).

**Gene expression microarray**

RNA extracted from paired RASF samples (n = 3) that had been treated 24 h transfected with NTC- or HDAC1-targeting-siRNA treated RASF was run on an Illumina HumanHT-12 v4 BeadChip (Illumina, CA, USA). Each array on the HumanHT-12 v4 Expression BeadChip targets >47 000 probes on a single BeadChip. For each probe represented on the array, beads are assembled with an average 15-fold redundancy. Total RNA was amplified using Illumina Total RNA Amplification kit from Ambion and 750 µg biotinylated cRNA was hybridized to the BeadChips at 58°C overnight (16–20 h) before being washed and stained according to the manufacturer’s instructions. BeadChips were scanned using an Illumina Bead Array Scanner and fluorescent hybridization signals were assessed with Illumina GenomeStudio software. For the preprocessing step, the data were first filtered, removing any probes not expressed in at least one sample in order to reduce the number of false positives. Transcripts were considered detected if the P-value comparing signal for a given transcript against negative controls was <0.05 for all hybridizations. The expression values were then adjusted with the variance stabilizing transformation method and then normalized with a quantile
normalization, both of which are used in the Lumi BioConductor package (Illumina). The groups were compared using the Bioconductor package Limma (Linear Models for Microarray Data) and the results were corrected for multiple testing using FDR. The FDR identify results using the P-value not the Fold Change. All of the microarray data have been submitted as a web supplement at the Gene Expression Omnibus repository of the National Center for Biotechnology Information (http://ubuntuone.com/7bctBQfAlxO6UB7AndA7kzu2).

**Gene ontology**
To obtain reproducible clusters for pathway analysis the data were analyzed with DAVID software. From the group of statistically significant genes showing differential expression we selected a group of candidate genes that were subsequently validated in the same RNA samples using SYBR green real-time PCR.

**Preparation of in vivo siRNA**
In vivo HPLC purified custom designed siRNA (Supplementary Material, Table S2), and predesigned NTC were purchased from Fisher Scientific (MA, USA). The in vivo siRNAs were diluted 1:1 with buffer (Fisher Scientific) and diluted 1:1 with Invivofectamine® (Fisher Scientific), incubated at 50°C for 30 min and dialyzed using a Float-a-lyser (VWR International, PA, USA) in sterile PBS at pH 7.4. The siRNA solutions were diluted in water to 0.5 mg/ml and stored at 4°C until needed. Of note, a vehicle control containing no siRNA was also prepared.

To confirm whether siRNA injection reduced HDAC1 expression in vivo 5 mg/kg of HDAC1 or NTC siRNA was administered intravenously. Seventy-two hours after injection, the tissue level of HDAC1 mRNA expression was measured by reverse transcriptase-polymerase chain reaction (RT-PCR) in the paws, major organs and peripheral blood.

**Collagen-induced arthitis mice**
Seven week-old male DBA/1 mice (Harlan, UK) were maintained under conventional animal housing conditions in a specific pathogen-free setting in accordance with the UK Animals (Scientific Procedures) Act, 1986. Mice were immunized intradermally into the base of the tail and subcutaneously in the flank with 50 μg/50 μl immunization grade Bovine Type II collagen solution (Chondrex, Inc., WA, USA) emulsified in complete Freund’s adjuvant (Sigma Aldrich). On Day 21 after the initial collagen immunization, the mice were intradermally boosted with 50 μg/50 μl bovine Type II collagen emulsified in Freund’s incomplete adjuvant (Sigma Aldrich). The severity of disease was graded using a scale of 0–4 for each of the four paws. Scoring was based on the severity and extent of erythema and edema of the periarthritic tissues (39). Treatment was initiated on Day 20 at which time the first animals were starting to show signs of inflammation. Mice were treated with either 5 mg/kg NTC or HDAC1-targeting siRNA (n = 10 both groups). The siRNA had been complexed with invivofectamine transfection reagent, then dialyzed prior to injection. Three intravenous injections were given separated by 1-day intervals.

**Histologic analysis**
Hind paws were fixed in formalin, decalcified over 2 weeks in EDTA buffer and embedded in paraffin. Ankle joint sections were stained with hematoxylin and eosin or Safranin O, scanned using an Aperio CS2 slide scanner (Leica) and analyzed manually. Hematoxylin and eosin (H&E) and Safranin O slides were scored manually from 0–3 (Grade 0 = normal, Grade 1 = slight, Grade 2 = moderate, Grade 3 = severe), for each sign of arthritis; synovial hyperplasia, inflammation, pannus formation and bone/cartilage loss (40). Safranin O slides were manually scored for the number of Safranin O expressing joints out of total synovial joints.

**X-ray microcomputed tomography analysis**
3D micro-tomodensitometry of mouse paws was performed using Skyscan 1172 high-resolution X-ray microcomputed tomography analysis (micro-CT) (Bruker, Kontich, Belgium) set to use a medium sized camera (2000 × 1024) at a resolution of 4.3 μm pixels. To quantify bone erosion within the hindpaws, 3D analysis CTAn software was used on a 2 mm length of the third metatarsophalangeal articulation.

**Statistical analysis**
A paired student t-test was used for in vitro studies of paired samples of RASF with P = 0.05 set as the limit of significance. For in vivo studies, two-way ANOVA with Bonferroni post hoc tests were performed. Results are expressed as the mean ± SD.

**Supplementary Material**
Supplementary Material is available at HMG online.

**Conflict of Interest statement.** None declared.

**Funding**
The work was supported by a PhD studentship (S.H.) from the University of Sheffield.

**References**


32. Ito, K., Yamamura, S., Essilfe-Quaye, S., Cosio, B., Ito, M., Barnes, P.J. and Adcock, I.M. (2006) Histone deacetylase 2-